APPARENT GENETIC MONOGAMY IN THE BEWICK'S WREN (*THRYOMANES BEWICKII*)

A Thesis

Presented to the

Faculty of the College of Graduate Studies and Research

Angelo State University

In Partial Fulfillment of the

Requirements for the Degree

MASTER OF SCIENCE

by

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May 2020

Major: Biology

APPARENT GENETIC MONOGAMY IN THE BEWICK'S WREN (*THRYOMANES BEWICKII*)

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ACKNOWLEDGEMENTS

I would first like to thank Dr. Ben Skipper for his guidance throughout my academic career at Angelo State University. I would not have been able to see this project through without his consistent support, assistance, and encouragement. I would like to thank Dr. Loren Ammerman for her assistance and guidance through implementing laboratory procedures and data interpretation necessary for the completion of this project. I would also like to thank Dr. Nicholas Negovetich for his involvement in my project.

I would like to thank the Angelo State University Department of Agriculture for allowing access to the Management, Instruction, and Research (MIR) Center and for granting me the permission to make repeated trips to sample wrens. I would also like to thank my sources of funding, specifically Angelo State University for the Angelo State Graduate Research Fellowship and the Angelo State University Department of Biology for the Head-of-the-River-Ranch travel grant.

I would like to thank my peers for their support throughout my graduate career. I would specifically like to thank my friends, Hannah Jones, Chastity Aguilar, Roxy Pourshoushtari, Sydney Decker, and Matt Hamilton for their assistance in the field or in the lab. I would also like to thank Jody Casares for her help and support.

Lastly I would like to give a special thanks to my family for their unconditional love and support throughout the entirety of my academic career.

ABSTRACT

Mixed reproductive strategies are common for many socially monogamous bird species. The levels of extra-pair paternity among passerine species are highly variable. Extra-pair paternity (EPP) has been documented for many socially monogamous birds including some members of the wren family (Troglodytidae), however, it has not been documented for the Bewick's wren (*Thryomanes bewickii*) although extra-pair reproductive behaviors have been suggested. I collected genotype data from 71 nestlings and 20 putative parents making up 13 broods. The rate of EPP was assessed with the use of 6 cross-species microsatellite loci. Of 13 broods, 12 (92.31%) contained no extra-pair offspring. Of 71 nestlings, 66 (92.96%) were sired by their putative fathers and 5 (7.04%) were assigned to candidate fathers not likely to be the true father. Bewick's wrens may follow a mixed reproductive strategy but remain mostly genetically monogamous, however, the result of EPP in 1 brood may be erroneous.

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INTRODUCTION

Parents are under intense selective pressure to maximize their fitness through the production of fit offspring. Pathways that maximize this fitness are many and include quantity-quality tradeoffs (Lack 1947), selective mate choice (Parker 2003), and bi-parental care (Cockburn 2006). Bi-parental care predominates in bird species and is expected when care from both parents is required for successful reproduction or when one sex cannot monopolize an essential resource needed for reproduction (e.g., nest sites or food). Although bi-parental care likely offers fitness benefits over uni-parental care, individuals within a pair could possibly increase their fitness further by soliciting copulations from individuals outside of the pair bond.

Studies of avian mating systems using genetic markers have shown that mixed reproductive strategies are common for many bird species and that true genetic monogamy is infrequent. To date, genetic polyandry has been documented in over 500 studies of more than 300 bird species (Brouwer and Griffith 2019). Among socially monogamous birds, extra-pair paternity has been detected in 76% of surveyed species (Brouwer and Griffith 2019). The number of extra-pair nestlings in a brood and proportion of broods with extra-pair nestlings is higher and more variable in passerine species than in other bird orders (Westneat and Stewart 2003), and no single, explanatory factor has been identified. However, the degree of gregariousness, divorce rate, sperm competition, and male ornamentation have all been positively correlated with variation in passerine extra-pair paternity, though there have been many exceptions (Quillfeldt et al. 2001).

Extra-pair copulation, and thus extra-pair paternity, is generally a result of females seeking to enhance the quality of heritable fitness for her offspring from different males based on sexual characteristics (Møller 1997). Aside from the direct material benefits that she may receive, females that engage in extra-pair mating may also receive the indirect benefit of genetic variability for her extra-pair offspring. However, this mating behavior may also be disadvantageous to females due to energy spent searching for a secondary mate, reduced parental investment (e.g., provisioning of young, nest defense) by the social mate, and risks of sexually transmitted diseases which may be fatal in some cases (Brouwer and Griffith 2019). Males who engage in extra-pair copulations will benefit from gaining additional fertilizations and thus increasing their fecundity without the investment into parental care (Forsman et al. 2008). Extra-pair paternity is best conceptualized as arising from a three-player game where each player's fitness is dependent upon the behaviors of the other players, in addition to social and ecological dynamics. The three players include extra-pair males, pair males, and females. Mate guarding by the pair male and the extent of extra-pair mate solicitation in females affect the ability of extra-pair males to gain extra-pair copulations. As a result, males should therefore invest in mate guarding and parental care to increase the certainty of paternity (Møller and Birkhead 1993).

The incidence of extra-pair young and factors explaining variation in extra-pair mating have been well studied in passerine birds. Black-capped chickadee (*Parus atricapillus*) females employ a mixed reproductive strategy to increase overall reproductive success by soliciting copulations from males with greater fitness (higher dominance) than the current mate. In such cases, up to 37.5% of offspring may be from outside the pair bond (Otter et al. 1994). Red-winged blackbirds (*Agelaius phoeniceus*) have been studied to

determine effects of breeding synchrony on extra-pair mating, where it was hypothesized that increased breeding synchrony should either promote extra-pair mating by enhancing the advantages of this behavior to females, or decrease extra-pair mating by preventing males from seeking extra-pair copulations. Results showed that although there was no variation of extra-pair paternity due to nesting synchrony, there was an advantage of breeding synchrony to males because of the affect it had on mate guarding. This effect increased the number of fertilizable females on a cuckolded male's (defined as a male who loses paternity to a rival) territory and decreased the number of these females on a cuckolder's (defined as a male that usurps paternity from a rival) territory (Weatherhead and Yezerinac 1997). Additionally, female red-winged blackbirds who engage in extra pair mating gain access to feeding territories at a higher rate than females who forego extra-pair mating (Gray 1997). Reed buntings (*Emberiza shoeniclus*) have evolved intense sperm competition in response to high levels of extra-pair mating (Dixon 1997). This species also has been studied to investigate the effects of local ecological factors on extra-pair mating behavior, where researchers found that colder weather conditions during peak fertile periods of the females increased levels of extra-pair paternity, which can partially explain variation among species (Bouwman and Komdeur 2006).

Occurrence of extra-pair paternity among many bird species also might be a result of the female's assessment of a male's honest advertisement of the potential quality of his offspring. These advertisements or signals are secondary sexual characters, such as male phenotypes that reflect the fitness of the male, where more prominent ornamentation signals enhanced fitness. For example, Møller et al. (1996) reported that large badge size in male house sparrows (*Passer domesticus*) was directly related to good body condition and immune

defense. McGraw et al. (2001) reported on sexual selection for the house finch (*Carpodacus mexicanus*) and found that that males with a brighter plumage fledged more offspring than drab males. Sung and Handford (2019) found a correlation between male song and reproductive performance in the savannah sparrow (*Passerculus sandwichensis*), where males with certain song attributes paired earlier and fledged more offspring than males without those same song attributes.

Wrens (family Troglodytidae) are a small group of sexually monomorphic passerines represented abundantly in New World habitats and scarcely in the Old World. Wrens are highly vocal and male wrens often possess a large song repertoire. Collectively, a wide array of mating systems has been observed across the family. Some species, such as the house wren (*Troglodytes aedon*), frequently engage in extra-pair copulations (Forsman et al. 2008). Across five studies, 28-38% of house wren broods contained at least 1 or more extra-pair young (Johnson 2014). Other species, such as the Carolina wren (*Thryothorus ludovicianus*), have been reported as socially and genetically monogamous (Haggerty et al. 2001). Buff-breasted wrens (*Cantorchilus leucotis*) are genetically monogamous as reported by Gill and Stutchbury (2005). Still others, such as the banded wren (*Thryophilus pleurostictus*) have shown low levels of extra-pair paternity, with extra-pair males siring 4% of offspring in 10% of nests (Cramer et al. 2011). Rufous-and-white wrens (*Thryophilus rufalbus*) similarly have shown low levels of extra-pair paternity with 2% of nestlings sired by an extra-pair male (Douglas et al. 2012). Of the 85 species of Troglodytidae, only 5 (6%) have been assessed for extra-pair mating behaviors. Given the diversity of mating strategies currently observed across the family, assessment of more species is warranted to determine if extra-pair mating

is a general (ancestral) feature of this avian family or a derived condition of some members (i.e., *Troglodytes aedon*).

Bewick's wrens (*Thryomanes bewickii*), like other wrens are sexually monomorphic and often form socially monogamous pair-bonds when courting. Bewick's wrens are relatively short-lived secondary cavity nesters that are typically found in dense shrubland (Taylor 2003). The breeding season for Bewick's wrens extends from April to early August and social bonds are generally established between March and early April. Bewick's wrens are asynchronous breeders and females may lay multiple broods per breeding season (Kennedy and White 2013). Paired males and females may both construct the nest and are often found foraging together before the nesting cycle. Pairs often remain together for a subsequent brood and may lay more than 2 broods if early clutches fail (Kennedy and White 2013). Bewick's wrens are opportunistic secondary cavity nesters and the nests are cups or domes within the cavity no more than 10 m above ground. The average clutch size for Bewick's wrens ranges from 3-8 eggs, but on average, females lay 6 eggs. Biparental care in Bewick's wrens is prevalent as females incubate while males provision the incubating female and nestlings. The incubation period is extended over 14-16 days and all eggs hatch within the same day. Nestlings may fledge as early as day 12, but usually depart on days 14-16 (Kennedy and White 2013). Young may continue to be fed by parents two weeks post-fledge. The total nesting cycle lasts about 60 days. Males establish territorial boundaries 1-7m apart from neighboring males (Miller 1941), and average territory size ranges from 2.0-3.8 ha (Kroodsma 1973a). Although polygyny has been suggested as a possible mating behavior of Bewick's wrens (Kroodsma 1972; Kennedy and White 1996), evidence of extra-pair copulations or extra-pair young has not been reported. Bewick's wrens exhibit many life

history traits that are often associated with low levels of extra-pair paternity such as a low-density breeding population, asynchronous breeding, active mate-guarding, and bi-parental care. While those traits are suggestive of social and genetic monogamy, male Bewick's wrens do display a complex song repertoire which may be an honest advertisement of his genetic quality, an attribute common in polygynous mating systems (Hasselquist 1998).

Genetic analysis using microsatellite loci can be used to determine the levels of extra-pair paternity in a population. In an effort to increase the knowledge of social behaviors for this species, my objective was to determine the prevalence of extra-pair fertilizations using microsatellite markers in a population of Bewick's wrens breeding in managed rangelands of San Angelo, Tom Green County, Texas. My null hypothesis was that Bewick's wrens are both socially and genetically monogamous. I expect that because Bewick's wrens are sister to Carolina wrens (Barker 2017), frequency of extra-pair paternity will be nonexistent or low.

MATERIALS AND METHODS

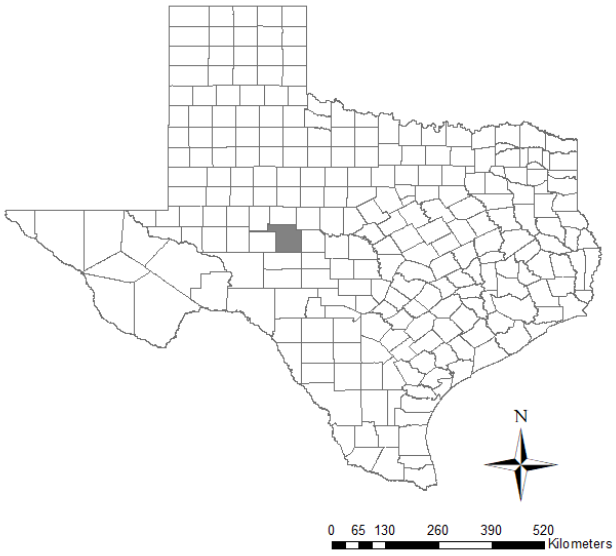
Study Area and Field Methods

The study was conducted between April and August of 2018 and 2019 on the Angelo State University Management, Instruction, and Research ranch in Tom Green County, Texas (Fig. 1). The landscape consists of dense shrub cover and grasslands actively used by livestock (cattle, goats, and sheep). To facilitate access to nestlings and parents, fifty nest boxes were provided in suitable areas of the ranch before the breeding season of 2018 and were readily used by Bewick's wrens. I constructed the nest boxes using a 20 cm segment of 10 cm diameter PVC pipe capped at both ends. Additionally, a 3.2 cm diameter hole was drilled into the surface of the PVC pipe for entry and exit of the wrens. I mounted the nest boxes on trees or free-standing conduit 1-1.5 m above the ground and spaced approximately 80 m apart. Nest boxes were spray painted with drab brown paint to simulate the appearance of natural cavities. Due to a high occurrence of nest predation in 2018, fifty additional nest boxes were provided before the breeding season of 2019.

With the help of field assistants, all nest boxes were monitored at 1 week intervals until signs of occupancy were noted (i.e., nest material). Once a nest box became occupied, I monitored occupied nest boxes every three days. To determine when nestlings would fledge, nests were monitored through the laying, incubating, and nestling periods of the nesting cycle. All nest checks were as brief and non-invasive as possible to decrease the possibility of nest abandonment. On nestling day 10 (day of hatch = 0), adult wrens were captured near their nests using mist nets. I considered the first male and female Bewick's wrens captured at a nest box to be the putative parents. Once the adults were captured, I marked them with a unique combination of colored leg bands for identification and association with individual

nest boxes. Measurements of the tail, tarsus, wing, mass, and bill dimensions were recorded. The sex of the wren was determined by the presence or absence of a cloacal protuberance and later confirmed by a molecular sex identification method. In addition, I collected no more than 100 µl of blood directly from the brachial or tarsal vein via venipuncture with a 26-gauge needle (Fair et al. 2010). After collecting blood, I placed an absorbent cotton ball firmly against the venipuncture wound to aid in blood clotting. Adults were released at the site of capture and blood was stored in lysis buffer (Longmire et al. 1988). On nestling day 12, I removed the nestlings from the nest box to be weighed, banded with a standard aluminum leg band, and bled following the protocol described above. Nestlings were returned to the nest shortly after collection of blood. Blood samples were stored in lysis buffer at room temperature until DNA extraction (Longmire et al. 1988). In the case of one brood and its attending adults, feather samples were used in lieu of blood. The Angelo State University IACUC (protocol 19-202) approved all capture and sampling techniques and banding was done under U. S. Geological Survey Bird Banding Laboratory permit number 22801 and Texas Parks and Wildlife Department state scientific permit number 1215-258.

Figure 1. Map of Texas with location of the study in Tom Green County highlighted.



Molecular Procedures

I isolated DNA from blood samples for 12 family groups and from feathers for 1 family group using standard protocols from the DNeasy Blood and Tissue kits (Qiagen, Inc., Valencia, CA). I identified a total of 23 cross-species microsatellite loci used in other studies of extra-pair paternity (Table 1). Of the 23 loci, 10 produced a polymerase chain reaction (PCR) product and 6 of these were variable, meaning that they possessed more than 2 alleles, so these 6 were used in paternity analysis for the Bewick's wren. 13 microsatellite loci were excluded from my study due to amplification failure and 4 were excluded due to extremely low allelic variability (Table 1). I altered PCR protocols and reactions to attempt to optimize the 13 microsatellite loci that failed to amplify and upon many trials of optimization attempts, these loci failed to amplify. Each wren was genotyped at 6 autosomal cross-species microsatellite loci (TA-B4-2, TA-A5-2, TA-C3(B)-2, ThP1-01, ThP1-17, and ThP1-30) using the Type-it Microsatellite PCR Kit (Qiagen Inc.) (Table 2). The PCR reactions

contained: 6.25 µl of master mix, 0.625 µl of 10 µM fluorescent-labeled forward primer (WellRED fluorescent dye; Sigma-Aldrich Corp., St. Louis, Missouri), 0.625 µl of 10 µM reverse primer (AlphaDNA, Montreal, Quebec, Canada, or Sigma-Aldrich Corp., St. Louis, Missouri), 4.0 µl sterile water, and 1 µl of template DNA (5-10 ng). The thermal cycler profile included an initial denaturing step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, annealing temperature (primer specific, Table 2) for 1 min, and extension at 72°C for 30 sec. A final extension of 60°C for 30 min completed the profile. Two sets of primers were multiplexed. The pairs included: TA-A5-2 with TA-B4-2, and ThP1-17 and ThP1-01 with ThP1-30.

I followed the protocol described by (Griffiths et al. 1998) to identify the sex of the nestlings and confirm the sex of adults. This method employed two primers, P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'-CTCCCAAGGATGARAAYTG-3') that anneal to conserved coding regions and amplify a non-coding intron in CHD (chromo-helicase-DNA-binding) genes on W and Z chromosomes. Males are the homogametic sex and females are heterogametic. PCR products were separated by electrophoresis and visualized on a 1.5% agarose gel that was stained with ethidium bromide. Nestling sex ratio data were analyzed in R using a binomial test (Krackow and Tkadlec 2001).

Table 1. Cross-species microsatellite loci excluded from Bewick's wren paternity analysis. Loci marked with * produced a PCR product but were not variable.

Locus	Primer Sequence	Repeat motif	Reference author
Troglodytes aedon			
TA-A5-15	F: CAACACAAGGTATCAATGAAGAGC R: CAAAAGGTGCCATTGCCTAT	$(TG)_{10}$	Cabe and Marshall (2001)
TA-C6-7*	F: TGCAGTAGAAGACAGAGAGTAGCA R: GCACAGCTGAGGTGATCTTG	$(AC)_2GT(AC)_7$	Cabe and Marshall (2001)
HrU3	F: CACTGGCTCTAGGCTGTCATC R: CTGTCCCATGTCAGGCCAGTC	$(CA)_{13}((A)_n(T)_n)_5$	Johnson et al. (2002)
HrU6	F: GCTGTGTCATTTCTACATGAG R: ACAGGGCAGTGTTACTCTGG	$(AAAG)_{17}(AG)_2(AAAG)_2$	Johnson et al. (2002)
POCC1	F: TTCTGTGCTGCAATCACACA R: GCTTCCAGCACCACTTCAAT	$(CA)_{13}(CG)_3G(CA)_9$	Johnson et al. (2002)
FHU2	F: TGATCGAAAGACCTGTAAGAT R: ATCAGCGTTAGACCAATACTCTTA	$(TC)_8$	Johnson et al. (2002)
PCAU3	F: GGTGTTTGTGAGCCGGGG R: TGTTACAACCAAAGCGGTCATTTG	$(GT)_6CT(GT)_3CT(GT)_5CT(GT)_3CT(GT)_{13}$	Johnson et al. (2002)
Chiroxiphia linearis			
LTMR6	F: GCCATGCCACAGGAGTGAGTC R: AGTCATCTCCATCAAGGGCAT	Dinucleotide repeat	McDonald and Potts (1994)
Malurus cyaneus			
McyU4	F: ATAAGATGACTAAGGTCTCTGGTG R: TAGCAATTGTCTATCATGGTTTG	$(GT)_{26}AT(GT)_3$	Double et al. (1997)
Thryophilus pleurostictus			
ThP1-14	F: GTAAATTTCAGGAGTCCAGGTTGC R: AAGCGCCCAAAATTAGCCAGAA	$(CA)_5(GACATACAGA)(CA)_7$	Brar et al. (2007)
ThP1-15*	F: TTGTCTTCTTCTCAGTTTGTCTCA R: GCGTTTGTGTTACTGAAGATTTAG	$(GT)_2(GA)(GT)_4$	Brar et al. (2007)
ThP1-16	F: CACTCTTTGAATTAGCTCTCCTCA R: GCAAAAACAAGATATCCTCAGTCC	$(TG)_8$	Brar et al. (2007)
ThP1-20*	F: CTTGCCATAGAATGCAGTTGAAT R: TAGTTCCAGTCCTCTTCTTTTACC	$(GT)_3GA(GT)_7$	Brar et al. (2007)
ThP1-22	F: GAGAAGAGTGCATAGGACAATCA R: TGGTGGCACGTTACAGGTTT	$(GT)_1(CT)_1(GT)_2(AT)_1(GT)_4(CT)_1(GT)_3$	Brar et al. (2007)
ThP1-26*	F: TCAAATGTGCCACTGACTGAGT R: AGCCTACTTCAAACTGAGACAGA	$(GT)_8$	Brar et al. (2007)
ThP1-27	F: TCTCTGCGTCTGCTTGGTG R: CTTCCTGGGATAGATAATGTGAC	$(AC)_{15}$	Brar et al. (2007)
ThP1-37	F: CCATCAGATTCCTAAGATAAACC R: TCCCAGCCCACCCTGTA	$(CAA)_7$	Brar et al. (2007)

Table 2. Six cross-species microsatellite loci used in Bewick's wren paternity analysis.

Species/Locus	Primer Sequence	Repeat motif	T_A (°C)	Size range (bp)	Reference author
Troglodytes aedon					
TA-B4-2	F: GCCTTCCTTACCCGTTGG R: TGGCAGAAATTCTGGCTGT	$TGTC(TG)_8$	55°C	167-175	Cabe and Marshall (2001)
TA-A5-2	F: TCTGGGAGGTCTCTCTCTAA R: TAGGAGAGGAGGGAGAGC	$(AC)_7(AN)_3(AC)_3AT(AC)_2$	55°C	162-196	Cabe and Marshall (2001)
TA-C3(B)-2	F: CCTGTGCCAGTGCTTTTCTT R: CCTTGGTCAGCTCATGGAAT	$(GT)_{19}N_{10}(TG)_2$	56°C	197-233	Cabe and Marshall (2001)
Thryophilus pleurostictus					
ThP1-01	F: CTTTGGGGCAGTGTTGTGGAATG R: GGCTGGCTGGGAGGCACAG	$(GT)_8$	62°C	302	Brar et al. (2007)
ThP1-17	F: AGTGGCTGGGTGTTCTTTCAT R: CACATCCTTTCCCTCCTGGTA	$(GT)_8$	56°C	157	Brar et al. (2007)
ThP1-30	F: ATGCCAGCACTAAAGAATGACAA R: CTACATAGCAGGCAGCAGAGGTT	$(TG)_6TA(TG)_3$	60°C	221	Brar et al. (2007)

Genotyping

The WellRED fluorescently labeled microsatellite products were separated on a Beckman Coulter CEQ8000 DNA Analysis System using a 400 base-pair size standard (AB Sciex, Concord, Ontario, Canada). Allele sizes were determined by using the chromatograms to visualize each fragment of DNA. I did not score peaks with signal strengths below 5000 units of fluorescence. I randomly selected 10 of 91 samples (10.99%) to be reamplified at all 6 loci and scored again to ensure consistency of genotyping methods. 91.7% (55 of 60 total reactions) successfully reamplified identical genotypes. Genotypes that differed were run a third time to dispute disagreements. Although most individuals were successfully scored at all 6 loci, 1 adult female was only scored at 5 of the 6 loci because one locus (TA-C3(B)-2) did not amplify. As a result, 0.3% of genotyping data were missing from the full data set used in parentage analyses.

Parentage Analysis

I used Cervus 3.0 (Kalinowski et al. 2007; Forsman et al. 2008), to quantify the number of alleles, observed and expected heterozygosity, polymorphic information content, non-exclusion probability, and deviation from Hardy-Weinberg equilibrium at each locus based on the genotypes of 91 individuals at 6 microsatellite loci. Cervus 3.0 also was used to estimate parentage which uses a maximum likelihood approach that accounts for genotyping error. Parentage was first assessed by manually identifying allelic mismatches across family groups. Parentage was then analyzed in Cervus 3.0 by comparing the genotypes of all adults to each nestling's genotype to determine if the putative parents would be assigned to their presumed offspring. The most likely parent for both maternity and paternity were based on pair LOD (Log of Odds) scores with a strict confidence level of 95% and a relaxed

confidence level of 85%. The value of LOD is the natural log of the overall ratio between the probability that the putative parent is the true parent and the probability that any other adult at random is the true parent. Pair LOD scores represent the log-likelihood ratio for a parent-offspring relationship between the known parent and the offspring. A positive pair LOD score indicates that the putative parent is more likely to be the true parent than any other adult in the population at random. A negative pair LOD score represents mismatches of genotypes at one or more loci and indicates that the putative parent is not likely to be the true parent. The critical values of LOD for both maternity and paternity was estimated in Cervus 3.0 prior to parentage analysis based on the genotype data of all adults and 10,000 simulated offspring at 5 microsatellite loci due to the small percentage of missing data at 1 locus. To assign paternity, I used the social mother as the known parent and included all adult males that were genotyped to represent a pool of candidate fathers.

RESULTS

I collected blood samples and genotyped a total of 71 Bewick's wren nestlings and 20 adults making up 13 broods in 2018 and 2019. Average brood size was 5.46 (SD=1.55). The sex ratio of the 71 nestlings was 37 males to 34 females (Fig. 2). The frequency of males within the sampled population (52.11%) was not significantly different than the expected binomial distribution of 50% (P=0.813). The sex of all parents (positive controls) were correctly identified. Based on the genotypes of 91 individuals, the average number of alleles per locus was 13.833 with the lowest number of 3 alleles at locus TA-B4-2 and the highest of 24 alleles at locus ThP1-30 (Table 3). Observed heterozygosity ranged from 0.538 to 0.967 and on average, expected heterozygosity was 0.812. Polymorphic information content (PIC) is a measure directly related to the ability of a locus to detect genetic polymorphisms and can range from 0 to 1.0. The mean polymorphic information content was 0.786. Of the 6 microsatellite loci analyzed, Cervus 3.0 did not perform a Hardy-Weinberg test on 2 loci. This issue is the result of an insufficient sample size at a locus, specifically when the number of genotypes multiplied by the frequency of the rarest allele squared equals less than the expected frequency. Of the 4 remaining loci, 2 were in Hardy-Weinberg equilibrium (Table 3). Combined non-exclusion probabilities for first parent and second parent were no greater than 0.007, meaning that it is unlikely that the microsatellite loci will assign an unrelated candidate parent even if the genotype of the other parent is not known. Paternity was assigned to 66 of 71 nestlings in 13 broods of the Bewick's wren. Five of 71 nestlings were assigned to candidate fathers with negative pair LOD scores.

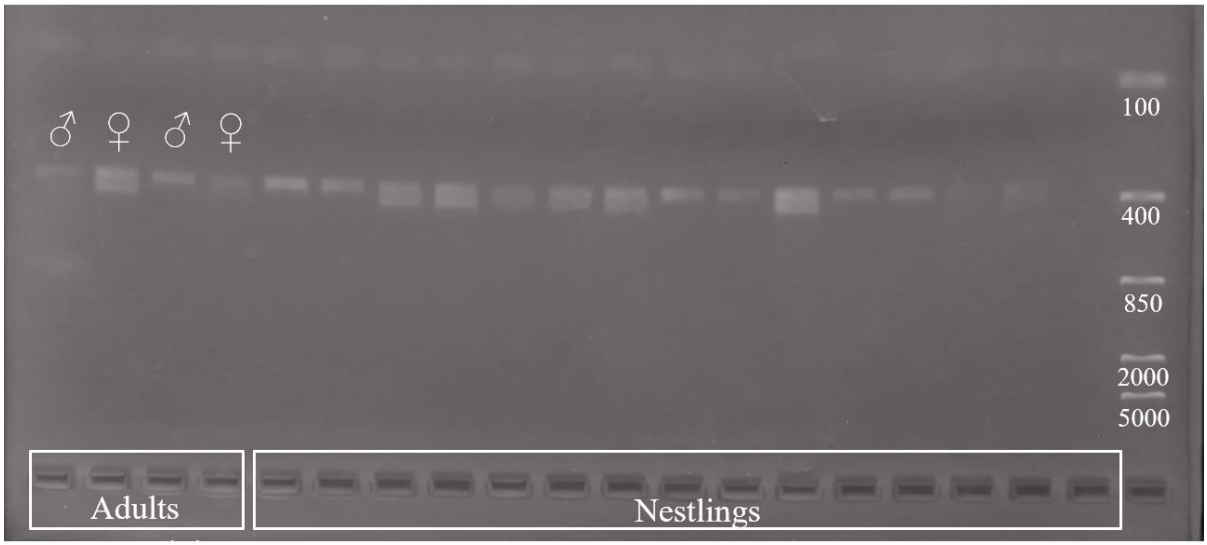


Figure 2. Sample of molecular determination of the sex of individual Bewick's wrens after separation of PCR products on a 1.5% agarose gel stained with ethidium bromide. Samples were amplified with P2/P8 primers of male and female Bewick's wrens. Positive controls are adult Bewick's wrens.

Table 3. Polymorphic parameters of microsatellite primers used in Bewick's wren paternity analysis estimated in CERVUS 3.0 based on genotype data from 91 individuals. The number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosity, polymorphic information content (PIC), probability of non-exclusion for first (P_{nex1}) and second (P_{nex2}) parent, and estimated null allele frequency (F_{null}). Loci with * deviated from Hardy-Weinberg equilibrium.

Locus/Species	N_A	H_O	H_E	PIC	P_{nex1}	P_{nex2}	F_{Null}	Reference author
Troglodytes aedon								
TA-B4-2	3	0.538	0.482	0.408	0.885	0.771	-0.0747	Cabe and Marshall (2001)
TA-A5-2*	14	0.967	0.862	0.844	0.435	0.276	-0.0665	Cabe and Marshall (2001)
TA-C3(B)-2	13	0.667	0.848	0.83	0.459	0.295	0.1077	Cabe and Marshall (2001)
Thryophilus pleurostictus								
ThP1-01*	10	0.593	0.821	0.796	0.524	0.350	0.1638	Brar et al. (2007)
ThP1-17	21	0.890	0.934	0.924	0.250	0.143	0.0219	Brar et al. (2007)
ThP1-30	24	0.912	0.927	0.917	0.268	0.155	0.0052	Brar et al. (2007)

Paternity analysis revealed that of the 13 broods from 2018 and 2019, 12 (92.31%) showed no evidence of extra-pair paternity. Of the 71 nestlings, 66 (92.96%) were assigned to their social fathers with positive pair LOD scores, and 5 (7.04%) were assigned to candidate fathers with negative pair LOD scores, indicating that the most likely candidate father was not the true father. Paternity for these 5 nestlings could not be assigned, however, these results agree with what I found when manually comparing the genotypes of offspring to the genotypes of their putative parents (Table 4). I found that all 5 nestlings in 1 brood had allelic mismatches when compared to their social father at 2 of the 6 loci (TA-A5-2 and ThP1-30). The mismatched alleles at those 2 loci were not found in any other individual male sampled in the population.

Table 4. Genotypes of brood with allelic mismatches among nestlings and putative father in the Bewick's wren. Band numbers were used for individual identification.

		Microsatellite loci					
Individual	Sex	TA-A5-2	TA-B4-2	TA-C3(B)-2	ThP1-01	ThP1-17	ThP1-30
Adults							
2311-21654	F	175/207	164/166	187/196	298/298	187/187	264/274
2311-21637	M	205/205	166/166	194/200	298/300	212/224	239/245
Nestlings							
2311-21649	F	175/175	164/166	196/196	298/298	187/224	241/264
2311-21650	M	207/213	164/166	194/196	298/300	187/210	257/264
2311-21651	F	175/202	166/166	194/196	298/298	187/224	250/274
2311-21652	M	202/207	164/166	196/196	298/298	187/224	250/265
2311-21653	M	207/213	164/166	187/194	298/298	187/224	258/264

DISCUSSION

I found a low frequency of extra-pair paternity in the Bewick's wren. Of the sampled nestlings, 7.04% (5 of 71) were not assigned to their putative father in 7.69% (1 of 13) broods. All nestlings whose genotypes did not match with their putative father in this study came from the same brood. The conclusion of EPP in this brood is based on the results of two loci. At one locus (TA-A5-2) there were two new alleles in the nestlings that could not have been contributed by the putative father (Table 4). Thus, this locus suggests that another male with a genotype 202/213 was the father. The second locus that suggests EPP is more problematic (ThP1-30). Collectively there were 5 alleles in the offspring that were not present in either of the putative parents and one of the nestlings did not have the alleles of the social mother (Table 4). There are several possible explanations for the apparent extra-pair paternity detected in this brood. First, this locus had the highest allelic diversity in the study which could be the result of a complex repeat motif type (Table 2) that is more prone to mutational error during PCR. However, this was not problematic for any of the other broods included in my study. Second, because this primer set was developed for another genus of wren (*Thryophilus pleurostictus*), perhaps it did not amplify the correct target region in the Bewick's wren genome and homologous DNA fragments were not analyzed. This is always a potential problem when using cross-species amplification but usually the result is that the loci exhibit ascertainment bias and are less variable (Delport et al. 2006). However, other studies of the same genus of wren have used this locus (Douglas et al. 2012). It has been observed that there is a decrease in proportion of loci that are successful in cross-species amplification with increasing phylogenetic distance between the source and target species

(Primmer et al. 1996). Lastly, it is possible that there were multiple fathers to this brood and none of them were the father captured at the nest.

Another possibility for the allelic mismatches of all 5 offspring at some but not all loci is that I may have captured a male sibling from a previous year instead of the father at the nest. Cooperative breeding and specifically helping by young of the previous brood has been documented in some species of bird (Skutch 1999). Although helping has not been documented in Bewick's wrens, if this scenario was correct, this may explain the presence of allelic mismatch in this brood at some but not all loci. Perhaps we captured and sampled a passerby male at the nest, or a male who did not sire the young. In the event of early divorce due to predation or abandonment, maybe the offspring were sired by the females first mate and I captured the secondary mate.

The results of this study suggest that Bewick's wrens may follow a mixed-reproductive strategy but remain mostly genetically monogamous. This low frequency of extra-pair paternity is similar to what has been reported for members of the genus *Thryophilus.* Cramer et al. (2011) reported low rates of extra-pair paternity in the banded wren, where 4% of nestlings were sired by extra-pair males in 10% of nests. Rufous-and-white wrens exhibited low levels of extra-pair paternity with 2% of all nestlings being extra-pair young (Douglas et al. 2012). A closer relative to the Bewick's wren is the Carolina wren, and Haggerty et al. (2001) documented this species as both socially and genetically monogamous, as none of 84 nestlings were extra-pair. Together, both my data and existing studies on extra-pair paternity in the family Troglodytidae suggest that that low rates of extra-pair paternity is a common family trait. This conclusion agrees with Griffith et al. (2002) who reported that more than 50% of interspecific variation in extra-pair paternity

occurs at the family or order level. House wrens show high levels of extra-pair paternity so they may be exhibiting a more derived trait whereas other wrens exhibit the ancestral trait of low levels of extra-pair paternity.

Several factors affect the prevalence of extra-pair copulations and rates of extra-pair paternity in socially monogamous bird species. Broad ecological and behavioral factors that have been reported to have an influence on levels of extra-pair paternity include breeding population density (Westneat and Sherman 1997; Griffith et al. 2002), breeding synchrony (Stutchbury and Morton 1995), mate guarding (Komdeur et al. 1999), and bi-parental care (Westneat and Stewart 2003). Brouwer and Griffith (2019) proposed that other factors such as latitude, habitat complexity, migration, generation length, genetic structuring, and climatic variability have been suggested to affect levels of extra-pair paternity, however there is no clear evidence that variation of extra-pair paternity can be explained by these variables.

In the presence of a highly dense breeding population, males and females may be more likely to engage in extra-pair copulation merely because the likelihood of encountering other breeding adults increase. Therefore, birds in a low-density breeding population may be constrained from engaging in promiscuous mating behaviors as opportunities to encounter other breeding adults decrease. For this study, I sampled 20 different adult Bewick's wrens during the breeding seasons of 2018 and 2019. Assuming that I sampled at least half of the breeding population, Bewick's wrens occur in a low-density breeding population, which is typical for species that exhibit genetic monogamy or low levels of extra-pair paternity. Nest boxes were not in close spatial proximity (about 80 m apart and 10 boxes per transect) in order to achieve and represent a natural density. In the event of an experimentally highly dense breeding population of Bewick's wrens, perhaps the opportunity to seek extra-pair

copulations would be more convenient which could yield higher levels of extra-pair paternity.

Stutchbury and Morton (1995) proposed that extra-pair paternity occurs at a higher frequency when females synchronously breed because they can simultaneously assess male quality. The alternative hypothesis is that breeding synchrony should decrease the rate of extra-pair paternity because males are occupying their time with mate guarding and parental care (Griffith et al. 2002). However, possible affects due to both density and breeding synchrony are contingent upon male and female behaviors respectively regarding mate guarding and active pursuits of extra-pair mates (Westneat and Stewart 2003). Given the low extra-pair paternity observed in my study and the fact that Bewick's wrens are asynchronously breeding birds, my results support the alternative hypothesis and suggest that extra-pair paternity rate is low in asynchronously breeding populations. Further, if males actively guard mates, the opportunities for extra-pair mating may be further reduced.

Mate guarding is a male behavior that is positively associated with certainty of paternity (Brouwer and Griffith 2019). For males to increase their overall reproductive success, most will engage in extra-pair copulations, but they should also prevent their social mate from doing the same. Males may also displace other males' sperm by sperm competition, in which the displaced male has a lower sperm count (Lifjeld et al. 1994). Komdeur et al. (1999) reported evidence that mate guarding during the female's peak fertility period also served as paternity assurance in Seychelles warblers (*Acrocephalus sechellensis*). I observed paired Bewick's wren males actively guarding their territories and their mates, specifically when nests were established. These behaviors are similar to what was reported by Kroodsma (1973b), where he found that paired males advertise by song during early

morning but follow their social mates around before she began incubating. My results are in agreement with what Brouwer and Griffith (2019) reviewed, because Bewick's wren males actively guarded their mates, they showed low levels of extra-pair paternity. In addition, my results suggest high mate fidelity in Bewick's wrens as 2 out of 4 adult breeding pairs from 2018 also formed broods in 2019.

Bi-parental care is common among socially monogamous bird species (Cockburn 2006). Female Bewick's wrens incubate and males provision the incubating female. Both parents feed nestlings, however nestling abandonment by male or female has been reported (Kennedy and White 2013). If the male does not abandon the nest, we can assume that males dedicate much of their time to parental care versus searching for extra-pair females. Females who do not seek extra-pair copulations should, in return, receive more help from her social mate. If her mate suspects that she engages in promiscuity, she may run the risk of abandonment. Investing in parental care for males is costly, so certainty of paternity should be positively associated with this behavior (Møller and Birkhead 1993). I captured Bewick's wren males at the nest shortly after deploying mist nets, which suggests that they were actively and closely monitoring the nest. This behavior may suggest that the males do invest in their reproductive efforts, however my data present limitations as I did not attempt to catch them at the nest early on in the nesting cycle. Perhaps they only increase their investment in bi-parental care during the late periods of nesting.

Bewick's wrens are relatively small birds, and smaller-bodied organisms generally have shorter lifespans in comparison to larger organisms (Lindstedt and Calder 1976). Because short-lived organisms necessarily have fewer breeding opportunities than long-lived organisms, it is likely that these groups have different strategies to maximize fitness. It has

been suggested that females of species with longer lifespans should invest in seeking out a male of high genetic quality because they have the time to do so. Furthermore, females of short-lived species should be more likely to mate with an extra-pair male of lower quality than her social mate due to time constraints (Wink and Dyrcz 1999). However, my results represent the contrary. Bewick's wrens exhibit low levels of extra-pair paternity and high mate fidelity, two attributes that are regularly associated with long-lived species. Because short-lived species should engage in extra-pair mating behaviors, perhaps Bewick's wrens are not short-lived. Similarly, Schmoll et al. (2009) found that males within-pair offspring recruits had a higher fecundity than male extra-pair offspring recruits in the coal tit (*Periparus ater*). Perhaps increased mortality rates of offspring may be offset by an increase in bi-parental care, yielding higher fecundity rates in within-pair offspring recruits. Because investment in pair bonds and paternal care are both positively associated with paternity assurance, this pathway may be selected for over investing in extra-pair copulations.

Another life history trait that may affect levels of extra-pair paternity is male ornamentation pertaining to sexual selection (Canal et al. 2011). Bewick's wrens are a sexually monomorphic species, however males advertise a large song repertoire. Male ornamentation in the form of song may be another honest advertisement of genetic quality for females to assess (Searcy 1992), so it is perplexing why male Bewick's wrens possess such a variable song if they do not regularly solicit extra-pair copulations, although polygynous behavior has been suggested (Kennedy and White 2013).

This study represents the first assessment of extra-pair paternity in the Bewick's wren, and more specifically with the use of cross-species microsatellite markers. Although 1 of 13 broods in this study revealed evidence of extra-pair paternity, Bewick's wrens may be a

socially and genetically monogamous passerine. True genetic monogamy is rare (<25% of socially monogamous species surveyed as reviewed by Griffith et al. 2002), so ongoing analyses of paternity in Bewick's wrens should be a priority in future studies. For many bird species that exhibit extra-pair paternity including those within the family Troglodytidae, incidence of extra-pair paternity is high, and intensity is low. My results show a low incidence of 1 brood and high intensity with all 5 nestlings showing evidence of extra-pair paternity. If the brood that exhibited evidence of extra-pair paternity was not a result of error, Bewick's wrens show a low level of extra-pair paternity which is common for the family Troglodytidae. My data may suggest a mixed reproductive strategy, but because my results are inconclusive, I do not reject or accept the null hypothesis of true genetic monogamy in the Bewick's wren. Because I only surveyed 71 birds making up 13 broods, my results may be insufficient to serve as a thoroughly representative dataset, as a sample size of 200 birds in an extra-pair paternity study has been recommended (Griffith et al. 2002). Despite my small sample size, this study provides information regarding life history traits in Bewick's wrens that have not been previously described.

LITERATURE CITED

Barker KF. 2017. Molecular phylogenetics of the wrens and allies (Passeriformes: Certhiodea), with comments on the relationships of *Ferminia*. American Museum Novitates. Number 3887.

Bouwman KM, Komdeur J. 2006. Weather conditions affect levels of extra-pair paternity in the reed bunting (*Emberiza schoeniclus*). Journal of Avian Biology. 37:238-244.

Brar RK, Schoenle LA, Stenzler LM, Hall ML, Vehrencamp SL, Lovette IJ. Eleven microsatellite loci isolated from the banded wren (*Thryothorus pleurostictus*). Molecular Ecology Notes. 7:69-71.

Brouwer L, Griffith SC. 2019. Extra-pair paternity in birds. Molecular Ecology. 28:4864-4882.

Cabe PR, Marshall KE. 2001. Microsatellite loci from the house wren (*Troglodytes aedon*). Molecular Ecology Notes. 1:155-156.

Canal D, Potti J, Dávila JA. 2011. Male phenotype predicts extra-pair paternity in pied flycatchers. Behavior. 148:691-712.

Cockburn A. 2006. Prevalence of different modes of parental care in birds. Proceedings of the Royal Society. 273:1375-1383.

Cramer ERA, Hall ML, De Kort SR, Lovette IJ, Vehrencamp SL. 2011. Infrequent extra-pair paternity in the banded wren, a synchronously breeding tropical passerine. Condor. 113:637-645.

Delport W, Grant TJ, Ryan PG, BloomerP. 2006. Ten microsatellite loci for evolutionary research on Nesospiza buntings. Molecular Ecology Notes 6:1180–1183.

Dixon A. 1997. Reproductive anatomy of the reed bunting: a species which exhibits a high degree of sperm competition through extra-pair copulations. Condor. 99:966-969.

Double MC, Dawson D, Burke T, Cockburn A. 1997. Finding fathers in the least faithful bird: a microsatellite-based genotyping system for the superb fairy-wren (*Malurus cyaneus*). Molecular Ecology. 6:691-693.

Douglas SB, Heath DD, Menhill DJ. 2012. Low levels of extra-pair paternity in a Neotropical duetting songbird, the rufous-and-white wren (*Thryothorus rufalbus*). Condor. 114:393-400.

Fair J, Paul E, Jones J. 2010. Guidelines to the use of wild birds in research. Washington D.C.: Ornithological Council.

Forsman AM, Vogel LA, Sakaluk SK, Johnson BG, Masters BS, et al. 2008. Female house wrens (*Troglodytes aedon*) increase the size, but not immunocompetence, of their offspring through extra-pair mating. Molecular Ecology. 17:3697-3706.

Gill SA, Stutchbury BJM. Nest building is an indicator of parental quality in a monogamous, tropical wren *Thryothorus leucotis*. Auk. 122:1169-1181.

Gray EM. 1997. Female red-winged blackbirds accrue material benefits from copulating with extra-pair males. Animal Behavior. 53:625-639.

Griffith SC, Owens IPF, Thuman KA. 2002. Extra pair paternity in birds: a review of interspecific variation and adaptive function. Molecular Ecology. 11:2195-2212.

Griffiths R, Double MC, Orr K, Dawson RJG. 1998. A DNA test to sex most birds. Molecular Ecology. 7:1071-1075.

Haggerty TM, Morton ES, Fleischer RC. 2001. Genetic monogamy in Carolina wrens (*Thryothourus ludovicianus*). Auk. 118:215-219.

Hasselquist D. 1998. Polygyny in great reed warblers: a long-term study of factors contributing to male fitness. Ecology. 79:2376-2390.

Johnson LS, Bonnie G, Masters BS. 2002. Increased cuckoldry as a cost of breeding late for male house wrens (*Troglodytes aedon*). Behavioral Ecology. 13:670-675.

Johnson LS. 2014. House wren (*Troglodytes aedon*), version 2.0 In The Birds of North America (P. G. Rodewald, editor). Cornell Lab of Ornithology, Ithaca, New York, USA. https://doi.org/10.2173/bna.380.

Kalinowski ST, Taper ML, Marshall TC. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology. 16:1099-1106.

Kennedy ED, White DW. 1996. Interference competition from house wrens as a factor in the decline of Bewick's wrens. Conservation Biology. 10:281-284.

Kennedy ED, White DW. 2013. Bewick's Wren (*Thryomanes bewickii*), version 2.0. In The Birds of North America (A. F. Poole, Editor). Cornell Lab of Ornithology, Ithaca, NY, USA. https://doi.org/10.2173/bna.315.

Komdeur J, Kraaijeveld-Smit F, Kraaijeveld K, Edelaar P. 1999. Explicit experimental evidence for the role of mate guarding in minimizing loss of paternity in the seychelles warbler. Proceedings of The Royal Society. 266:2075-2081.

Krackow S, Tkadlec E. 2001. Analysis of brood sex ratios: implications of offspring clustering. Behavioral Ecology and Sociobiology. 50:293-301.

Kroodsma DE. 1972. Bigamy in the Bewick's wren? Auk. 89:185-187.

Kroodsma DE. 1973a. Coexistence of Bewick's wrens and house wrens in Oregon. Auk. 90:341-352.

Kroodsma DE. 1973b. Singing Behavior of the Bewick's wren: Development, Dialects, Population Structure, and Geographical Variation [dissertation]. Corvallis (OR): Oregon State University.

Lack D. 1947. The significance of clutch-size. Ibis. 89:302-352.

Lifjeld JT, Dunn PO, Westneat DF. 1994. Sexual selection by sperm competition in birds: male-male competition or female choice? Journal of Avian Biology. 25:244-250.

Lindstedt SL, Calder WA. 1976. Body size and longevity in birds. Condor. 78:91-94.

Longmire JL, Lewis AK, Brown NC, Buckingham JM, Clark LM, et al. 1988. Isolation and molecular characterization of a highly polymorphic centromeric tandem repeat in the family Falconidae. Genomics. 2:14-24.

McDonald DB, Potts WK. 1994. Cooperative display and relatedness among males in a lek-mating bird. Science. 266:1030-1032.

McGraw KJ, Stoehr AM, Nolan PM, Hill GE. 2001. Plumage redness predicts breeding onset and reproductive success in the House Finch: a validation of Darwin's theory. Journal of Avian Biology. 32:90-94.

Miller EV. 1941. Behavior of the Bewick Wren. Condor. 43:81-99.

Møller AP, Kimball RT, Errizøe J. 1996. Sexual ornamentation, condition, and immune defense in the house sparrow *Passer domesticus*. Behavioral Ecology and Sociobiology. 39:317-322.

Møller AP. 1997. Immune defense, extra-pair paternity, and sexual selection in birds. Proceedings: Biological Sciences. 264:561-566.

Møller AP, Birkhead TR. 1993. Certainty of paternity covaries with paternal care in birds. Behavioral Ecology and Sociobiology. 33:261-268.

Otter K, Ratcliffe L, Boag PT. 1994. Extra-pair paternity in the black-capped chickadee. Condor. 96:218-222.

Parker TH. 2003. Genetic benefits of mate choice separated from differential maternal investment in red junglefowl (*Gallus gallus*). Evolution. 57:2157-2165.

Primmer CR, Møller AP, Ellegren H. 1996. A wide-range survey of cross-species microsatellite amplification in birds. Molecular Ecology 5:365–378.

Quillfeldt P, Schmoll T, Peter HU, Epplen JT, Lubjuhn T. 2001. Genetic Monogamy in Wilson's storm-petrel. Auk. 118:242-248

Searcy WA. 1992. Song repertoire and mate choice in birds. American Zoology. 32:71-80.

Schmoll T, Schurr FM, Winkel W, Epplen JT, Lubjuhn T. 2009. Lifespan, lifetime reproductive performance and paternity loss of within-pair and extra-pair offspring in the coal tit *Periparus ater*. Proceedings of The Royal Society. 276:337-345.

Skutch AF. 1999. Helpers at birds' nests: A worldwide survey of cooperative breeding and related behavior. Iowa (IA): University of Iowa Press.

Stutchbury BJ, Morton ES. 1995. The effect of breeding synchrony on extra-pair mating systems in songbirds. Behavior. 132:675-690.

Sung HC, Handford P. 2019. Song characters as reliable indicators of male reproductive quality in the Savannah Sparrow (*Passerculus sandwichensis*). Canadian Journal of Zoology. 98:32-38.

Taylor RV. 2003. Factors influencing expansion of the breeding distribution of Bewick's wren into riparian forests of the Rio Grande in Central New Mexico. Southwestern Naturalist. 48:373-382.

Weatherhead PJ, Yezerinac SM. 1997. Breeding synchrony and extra-pair mating in red-winged blackbirds. Behavioral Ecology and Sociobiology. 40:151-58.

Westneat DF, Sherman PW. 1997. Density and extra-pair fertilizations in birds: a comparative analysis. Behavioral Ecology and Sociobiology. 41:205-215.

Westneat DF, Stewart IRK. 2003. Extra-pair paternity in birds: causes, correlates, and conflict. Annual Review of Ecology, Evolution, and Systematics. 34:365-396.

Wink M, Dyrcz A. 1999. Mating systems in birds: a review of molecular studies. Acta Ornithologica. 34:91-109.

APPENDICES

Appendix I, Table 5.—Genotypes of 13 Bewick's wren broods using 6 cross-species microsatellite loci. Band numbers were used for individual identification. Individuals with * are adult females and individuals with ** are adult males. Band numbers were not assigned to 6 nestlings. Genotype data were missing for one adult female at locus TA-C3(B)-2.

	Microsatellite Loci					
Individual	TA-A5-2	TA-B4-2	TA-C3(B)-2	ThP1-01	ThP1-17	ThP1-30
2311-21611	199/215	164/166	191/193	297/299	210/214	247/264
2311-21612	175/199	164/164	191/193	297/299	210/214	264/264
2311-21613	175/199	166/166	191/193	297/299	208/222	247/264
2311-21614	175/197	164/166	191/193	297/299	208/214	264/264
2311-21615	175/199	164/166	189/193	297/299	208/214	264/264
2311-21616	197/215	166/166	189/193	297/299	210/214	247/266
2311-21617*	197/199	164/166	191/193	297/297	208/210	247/264
2311-21618**	175/215	164/166	189/191	299/299	214/222	264/264
2311-21627**	175/205	164/166	191/195	292/300	201/212	239/256
2311-21628*	201/213	166/166	195/195	287/287	214/222	256/276
2311-21629	205/213	166/166	195/195	300/300	201/214	239/276
2311-21630	175/201	164/166	195/195	287/300	201/222	239/256
2311-21631	175/201	166/166	195/195	287/292	212/214	239/276
2311-21632	175/213	166/166	195/195	292/292	212/214	239/276
2311-21649	175/175	164/166	196/196	298/298	187/224	241/264
2311-21650	207/213	164/166	194/196	298/300	187/210	257/264
2311-21651	175/202	166/166	194/196	298/298	187/224	250/274
2311-21652	202/207	164/166	196/196	298/298	187/224	250/265
2311-21653	207/213	164/166	187/194	298/298	187/224	258/264
2311-21654**	175/207	164/166	187/196	298/298	187/187	264/274
2311-21637*	205/205	166/166	194/200	298/300	212/224	239/245
2311-21655	201/211	166/168	200/204	297/299	183/212	247/260
2311-21656	175/201	166/168	200/204	294/298	199/206	264/270
2311-21657	201/211	166/168	191/200	294/299	183/212	247/260
2311-21658	201/211	166/166	200/204	297/298	183/199	247/270
2311-21659	175/201	166/168	191/200	294/299	206/212	260/264
2311-21660	175/201	166/166	191/200	294/298	199/206	247/260
2311-21661	201/211	166/166	191/200	294/299	206/212	260/264
2311-21639*	201/201	166/166	191/204	298/299	199/212	247/264
2311-21662**	175/211	166/168	187/200	294/297	183/206	260/270
2311-21627**	175/205	164/166	191/195	292/300	201/212	239/256
2311-21628*	201/213	166/166	195/195	287/287	214/222	256/276
2311-21671	175/213	166/166	195/195	300/300	201/222	239/276
2311-21672	201/205	164/166	195/195	287/300	201/222	256/256
2311-21673	201/205	166/166	195/195	287/300	201/222	256/256

Table 5.— Continued

Individual	Microsatellite Loci					
	TA-A5-2	TA-B4-2	TA-C3(B)-2	ThP1-01	ThP1-17	ThP1-30
2311-21674	201/205	166/166	191/195	292/292	212/214	256/256
2311-21675	175/201	166/166	195/195	300/300	212/214	256/256
2311-21676	175/201	164/166	191/191	287/300	201/214	239/276
2311-21695	175/199	164/166	191/193	294/300	204/212	232/256
2311-21696	175/199	164/166	191/210	294/300	204/220	249/256
2311-21697	197/217	164/164	191/193	294/300	212/220	232/268
2311-21698	197/217	166/166	193/210	294/300	204/212	232/268
2311-21699	197/199	164/166	191/210	294/300	212/220	249/268
2311-21700	175/217	164/166	191/193	300/300	220/220	232/256
2311-21701*	199/217	164/166	193/210	300/300	204/220	232/249
2311-21702**	175/197	164/166	191/193	294/294	212/220	256/268
2311-21641**	175/207	164/166	191/206	298/298	216/226	237/272
2311-21677*	211/215	166/166	193/197	300/300	210/224	257/264
2311-21678	207/211	164/166	191/197	298/300	216/224	264/272
2311-21679	175/211	166/166	191/197	298/300	210/226	257/272
2311-21680	175/211	166/166	197/206	298/300	216/224	237/257
2311-21681	175/211	166/166	191/197	298/300	210/216	257/272
2311-21682	207/211	166/166	191/193	298/300	210/226	264/272
2311-21683	207/215	164/166	193/206	298/300	216/224	237/264
2311-21684	175/211	164/166	193/206	298/300	216/224	237/264
2311-21719	205/211	164/166	191/193	293/293	183/212	247/255
2311-21720	205/211	166/166	191/193	293/293	183/212	241/247
2311-21721	175/197	164/166	191/193	300/300	183/216	241/247
2311-21722	175/197	166/166	191/193	300/300	216/221	241/268
2311-21723	175/197	166/166	191/191	300/300	216/221	255/268
2311-21724**	175/211	164/166	191/191	300/300	183/221	241/255
2311-21725*	197/205	166/166	191/193	293/300	212/216	247/268
1921-40694*	205/211	164/166	191/194	299/299	207/216	247/257
1921-40695**	175/220	164/166	191/194	295/299	211/216	243/262
2311-21663	175/211	166/166	194/194	295/299	207/211	257/262
2311-21664	175/211	164/164	191/194	295/299	211/216	243/247
2311-21665	211/220	164/166	191/194	295/299	207/211	247/262
2311-21666	211/220	164/166	194/194	295/299	207/216	243/247
2311-21667	175/211	166/166	191/191	299/299	216/216	243/257
2311-21693*	199/205	164/164	185/191	300/300	203/211	245/257
2311-21694**	175/209	166/166	—	300/300	211/218	247/251
N1	199/209	166/166	185/185	300/300	203/211	251/257
N2	199/209	166/166	185/185	300/300	211/211	245/247
N3	199/209	166/166	185/185	300/300	203/211	247/257
N4	175/199	166/166	185/185	300/300	211/211	245/247
N5	175/199	166/166	185/185	300/300	203/218	245/247
N6	199/209	164/166	191/191	300/300	203/211	251/257

Table 5.— Continued

Individual	Microsatellite Loci					
	TA-A5-2	TA-B4-2	TA-C3(B)-2	ThP1-01	ThP1-17	ThP1-30
2311-21639*	201/201	166/166	191/204	298/299	199/212	247/264
2311-21662**	175/211	166/168	187/200	294/297	183/206	260/270
2311-21709	175/201	166/168	187/204	294/299	206/212	264/270
2311-21710	175/201	166/168	187/204	294/298	199/206	260/264
2311-21712	201/211	166/168	187/204	294/298	199/206	247/270
2311-21713	175/201	166/168	187/191	294/298	199/206	247/260
2311-21714	201/211	166/166	187/204	297/299	183/212	247/270
2311-21715	175/201	166/168	200/204	297/299	183/199	247/270
2311-21633*	201/205	166/168	191/191	300/318	183/201	249/257
2311-21634**	175/199	164/166	191/191	295/300	220/220	237/268
2311-21635	175/201	164/166	191/191	295/318	201/220	249/268
1921-40694*	205/211	164/166	191/194	299/299	207/216	247/257
1921-40695**	175/220	164/166	191/194	295/299	211/216	243/262
1921-40687	175/211	164/166	191/194	299/299	216/216	243/247
1921-40688	211/220	164/166	194/194	299/299	216/216	243/257
1921-40689	175/211	164/166	194/194	299/299	207/216	243/257
1921-40690	175/211	166/166	191/191	295/299	211/216	247/262
1921-40691	175/205	164/164	191/194	295/299	216/216	243/257
1921-40692	205/220	164/164	191/194	295/299	207/216	257/262
1921-40693	205/220	164/166	191/191	295/299	216/216	257/262

Appendix II, Table 6.—Parentage assignment of 71 Bewick's wren nestlings to candidate parents based on genotype data and pair LOD (Log of Odds) scores from parentage analysis in CERVUS 3.0. Pair confidence with * indicates that the candidate parent assigned is the true parent with a strict level of 95% confidence. Pair confidence with + indicates that the candidate parent assigned is the true parent with a relaxed level of 85% confidence. Pair confidence with – indicates that the candidate parent assigned is not likely to be the true parent.

Offspring ID	Candidate mother ID	Pair LOD score	Pair confidence	Candidate father ID	Pair LOD score	Pair confidence
2311-21611	2311-21618	5.61E+00	*	2311-21617	6.98E+00	*
2311-21612	2311-21618	4.36E+00	*	2311-21617	7.36E+00	*
2311-21613	2311-21618	3.42E+00	*	2311-21617	7.26E+00	*
2311-21614	2311-21618	4.02E+00	*	2311-21617	8.26E+00	*
2311-21615	2311-21618	6.95E+00	*	2311-21617	7.50E+00	*
2311-21616	2311-21618	2.87E+00	*	2311-21617	5.84E+00	*
2311-21629	2311-21627	6.01E+00	*	2311-21628	3.92E+00	*
2311-21630	2311-21627	5.16E+00	*	2311-21628	7.99E+00	*
2311-21631	2311-21627	5.61E+00	*	2311-21628	8.96E+00	*
2311-21632	2311-21627	6.30E+00	*	2311-21628	3.92E+00	*
2311-21649	2311-21654	8.32E+00	*	2311-21637	-9.37E+00	-
2311-21650	2311-21654	8.07E+00	*	2311-21677	-4.63E+00	-
2311-21651	2311-21654	8.98E+00	*	2311-21637	-3.92E+00	-
2311-21652	2311-21654	4.99E+00	*	2311-21637	-9.37E+00	-
2311-21653	2311-21654	8.63E+00	*	2311-21637	-4.60E+00	-
2311-21655	2311-21662	7.86E+00	*	2311-21639	4.09E+00	*
2311-21656	2311-21662	7.32E+00	*	2311-21639	5.34E+00	*
2311-21657	2311-21662	7.59E+00	*	2311-21639	2.25E+00	*
2311-21658	2311-21662	6.22E+00	*	2311-21639	5.95E+00	*
2311-21659	2311-21662	7.19E+00	*	2311-21639	2.32E+00	*
2311-21660	2311-21662	5.42E+00	*	2311-21639	4.11E+00	*
2311-21661	2311-21662	6.02E+00	*	2311-21639	3.00E+00	*
2311-21671	2311-21627	4.89E+00	*	2311-21628	4.54E+00	*
2311-21672	2311-21627	5.92E+00	*	2311-21628	8.68E+00	*
2311-21673	2311-21627	5.40E+00	*	2311-21628	9.36E+00	*
2311-21674	2311-21627	7.10E+00	*	2311-21628	1.59E+00	*
2311-21675	2311-21627	4.11E+00	*	2311-21628	2.28E+00	*
2311-21676	2311-21627	3.68E+00	*	2311-21628	2.19E+00	*
2311-21695	2311-21702	4.70E+00	*	2311-21701	6.97E+00	*
2311-21696	2311-21702	4.16E+00	*	2311-21701	9.00E+00	*
2311-21697	2311-21702	8.17E+00	*	2311-21701	7.84E+00	*
2311-21698	2311-21702	5.86E+00	*	2311-21701	9.75E+00	*
2311-21699	2311-21702	6.56E+00	*	2311-21701	7.75E+00	*

Table 6.—Continued

Offspring ID	Candidate mother ID	Pair LOD score	Pair confidence	Candidate father ID	Pair LOD score	Pair confidence
2311-21700	2311-21702	5.49E-01	+	2311-21701	8.88E+00	*
2311-21678	2311-21641	5.59E+00	*	2311-21677	4.91E+00	*
2311-21679	2311-21641	5.44E+00	*	2311-21677	6.06E+00	*
2311-21680	2311-21641	5.90E+00	*	2311-21677	5.95E+00	*
2311-21681	2311-21641	3.25E+00	*	2311-21677	6.06E+00	*
2311-21682	2311-21641	7.25E+00	*	2311-21677	4.22E+00	*
2311-21683	2311-21641	8.24E+00	*	2311-21677	5.11E+00	*
2311-21684	2311-21641	6.43E+00	*	2311-21677	3.43E+00	*
2311-21719	2311-21724	1.38E+00	*	2311-21725	5.90E+00	*
2311-21720	2311-21724	3.46E-01	+	2311-21725	6.58E+00	*
2311-21721	2311-21724	5.31E+00	*	2311-21725	3.59E+00	*
2311-21722	2311-21724	6.08E+00	*	2311-21725	5.39E+00	*
2311-21723	2311-21724	7.28E+00	*	2311-21725	4.80E+00	*
2311-21663	1921-40695	6.02E+00	*	1921-40694	5.64E+00	*
2311-21664	1921-40695	6.61E+00	*	1921-40694	4.17E+00	*
2311-21665	1921-40695	8.24E+00	*	1921-40694	5.32E+00	*
2311-21666	1921-40695	7.71E+00	*	1921-40694	5.93E+00	*
2311-21667	1921-40695	3.93E+00	*	1921-40694	4.42E+00	*
N1	2311-21694	7.59E+00	*	2311-21693	3.68E+00	*
N2	2311-21694	6.05E+00	*	2311-21693	4.31E+00	*
N3	2311-21694	5.36E+00	*	2311-21693	3.68E+00	*
N4	2311-21694	3.77E+00	*	2311-21693	4.31E+00	*
N5	2311-21694	5.02E+00	*	2311-21693	4.65E+00	*
N6	2311-21694	6.91E+00	*	2311-21693	6.53E+00	*
2311-21709	2311-21662	7.54E+00	*	2311-21639	4.16E+00	*
2311-21710	2311-21662	7.41E+00	*	2311-21639	5.34E+00	*
2311-21712	2311-21662	8.15E+00	*	2311-21639	5.27E+00	*
2311-21713	2311-21662	7.41E+00	*	2311-21639	3.43E+00	*
2311-21714	2311-21662	6.44E+00	*	2311-21639	4.77E+00	*
2311-21715	2311-21662	7.39E+00	*	2311-21639	5.05E+00	*
2311-21635	2311-21634	6.47E+00	*	2311-21633	7.66E+00	*
1921-40687	1921-40695	5.15E+00	*	1921-40694	5.20E+00	*
1921-40688	1921-40695	7.66E+00	*	1921-40694	6.05E+00	*
1921-40689	1921-40695	4.87E+00	*	1921-40694	7.06E+00	*
1921-40690	1921-40695	5.33E+00	*	1921-40694	2.60E+00	*
1921-40691	1921-40695	6.23E+00	*	1921-40694	5.81E+00	*
1921-40692	1921-40695	7.93E+00	*	1921-40694	6.82E+00	*
1921-40693	1921-40695	7.57E+00	*	1921-40694	4.77E+00	*

ANGELO STATE UNIVERSITY
College of Graduate Studies & Research
Institutional Animal Care & Use Committee

2-13-19

Dr. Ben R. Skipper
Assistant Professor, Dept. of Biology
Angelo State University
Member, Texas Tech University System
ASU Station #10890
San Angelo, TX 76909-0890

Dear Dr. Skipper:

Your proposed amendment for protocol #17-50-R, "The role of food limitation in the breeding cycle of Bewick's wrens" was reviewed by Angelo State University's Institutional Animal Care and Use Committee (IACUC) in accordance with the regulations set forth in the Animal Welfare Act and P.L. 99-158.

This amendment was approved, effective 02-11-2019 and has been renumbered as #19-202. Expiration of this protocol is three years from the original protocol approval date; an annual review and progress report form (www.angelo.edu/content/files/22583-iacuc-annual-review-progressreport) for this project is due no later than 02-11 of each year. If the study will continue beyond three years, you must submit a request for continuation before the current protocol expires.

Please remember to include the protocol number (19-202) in the subject line of all future communications with the IACUC regarding this protocol.

Sincerely,

Steven T. Brewer, Ph.D.
Assistant Professor,
Director, MS Program in Experimental Psychology
Co-Chair, Institutional Animal Care and Use Committee
Department of Psychology & Sociology
Angelo State University
Member, Texas Tech University System
ASU Station #10907
San Angelo, TX 76909-0907
Phone: 325-486-6124

Appendix III. —Approval letter received by Dr. Ben Skipper from Dr. Steven Brewer, Co-Chair of the Angelo State University Institutional Animal Care and Use Committee (IACUC) on 2 February 2019. This IACUC protocol (19-202) was for the handling and collection of blood from Bewick's wrens caught by mist nets in San Angelo, Texas.

BIOGRAPHY

Victoria Kristine Solis graduated from Wayland Baptist University of Plainview, TX in December 2016 with a B. S. in Biology. Her undergraduate thesis research focused on determining home range sizes and habitat use of the ladder-backed woodpecker (*Picoides scalaris*) in the Caprock Canyonlands. After graduating from Wayland Baptist University, Victoria began working on her master's thesis under her major advisor Dr. Ben Skipper at Angelo State University in August 2017.